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Established Cells

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【Document Name】 Specification

【Title of the Invention】 ESTABLISHED CELLS

【Claims】

【Claim 1】 An established cell derived from retinal capillary endothelial cells, which expresses a temperature sensitive SV40 large T-antigen gene, GLUT-1 transporter, and p-glycoprotein.

【Claim 2】 The established cell according to claim 1, having a deposition number of FERM BP-6507.

【Claim 3】 A method of establishing an immortalized cell which expresses a temperature sensitive SV40 large T-antigen gene, GLUT-1 transporter, and p-glycoprotein, the method comprising treating retinal capillary vessels of a transgenic animal into which a large T-antigen gene of SV40 temperature sensitive mutant tsA58 has been introduced with protease and subculturing the resulting cells to obtain immortalized cells.

【Claim 4】 The immortalized cell according to claim 3, wherein the transgenic animal is a rat.

【Claim 5】 An established cell which expresses a temperature sensitive SV40 large T-antigen gene, GLUT-1 transporter, and p-glycoprotein, the cell obtained by treating retinal capillary vessels of a transgenic animal into which a large T-antigen gene of SV40 temperature sensitive mutant tsA58 has been introduced with protease and subculturing the resulting cells.

【Detailed Description of the Invention】

【0001】

【Technical Field where the Invention Belongs】

The present invention relates to established cells derived from retinal capillary endothelial cells.

The established cells obtained by the present invention fo rm a monolayer of the retinal capillary endothelial cells which have inside-and outside polarity when culturing in a culture dis h. Therefore, the established cells are useful for predicting pe rmeation of drugs to the retina by the assessment of chemical up take into the retinal capillary endothelial cells, studying supp ly and metabolism of various factors and nutritions in the retin al parenchyma, studying the transport mechanism of permeation of selective materials which are present in retinal capillary endo thelial cells, studying toxicity of chemicals on retinal capilla ry endothelial cells, and so on. In addition, a blood retinal b arrier can be reconstructed in the test tube (in vitro) by cocul ture with Mueller cells which are a kind of glia cells. The cell lines of the present invention are therefore useful for cellula r level studies on screening drugs regarding the safety and effi cacy thereof, and developing methods for diagnosing and treating diseases relating to intraocular homeostatic maintenance and fu nctional disorders of retinal tissues.

【0002】

【Background Art】

Conventionally, tests for the assessment of safety and efficacy of drugs have mainly been conducted using animals. However, to avoid use of a large number of animals from the viewpoint of animal right, technologies using cultured cells for in-vitro assessment of safety and efficacy of drugs are

used on a practical level. For example, a technique of first testing using primary culture cells collected from living tissues or established culture cells which can infinitely proliferate, and then testing using animals is employed. The primary culture cells can initially proliferate very well, but the proliferation gradually declines as the subculture advances, and finally cells die out. This phenomenon is called cellular senescence. Furthermore, in addition to the fear that the characteristics of primary culture cells may differ each time they are collected from living tissues, the primary culture cells are said to change the characteristics as the subculture advances. Particularly, when the multiplication rate is very slow or when the cells are derived from a small organ, it is very difficult to obtain a sufficient amount of the primary culture cells for test. On the other hand, established culture cell which have acquired the capability of infinitely proliferating during subcultures of the primary culture cells can maintain stable characteristics. However, most of these cells no longer have part or all of the forms and functions possessed by the cells when they were in a living body. Therefore, it is difficult for such established cells to precisely reflect the original characteristics which the cell lines exhibited in the tissues from which they have been derived. In view of this situation, establishment of immortalized cells which can continuously maintain an active proliferation capability possessed by the primary culture cells without losing the characteristics inherently possessed by the cells during subculture, has been tried by transforming

the cells by introducing oncogenes such as ras and c-myc, E1A gene of adenovirus, large T-antigen gene of SV40 virus, HPV16 gene of human papillomavirus, and the like. Such immortalized cells which are derived from some organs already lose several functions at the time of introducing oncogenes or large T-antigen genes after preparation of a primary culture cell. Thus, acquisition of immortalized cells in the stringent meaning of holding an original function has been difficult. Preparing a primary culture cell and acquiring a cell line has been very difficult, particularly when the multiplication rate is very slow or when the cells are derived from a small organ.

【0003】

To overcome these problems, a method of establishing immortalized cells by applying a recently developed transgenic technology to individual animals has been proposed. Instead of introducing oncogenes or large T-antigen genes into individual cells, according to this method, transgenic animals into which these genes have been introduced in chromosomes in a stable manner are prepared. Then, a primary culture cell is prepared from an organ of these animals which possesses the oncogenes or large T-antigen genes in the cells at the time of development of the individuals. The primary culture cells is subcultured to establish immortalized cells. In particular, immortalized cells are easily available from organs of transgenic mice into which a large T-antigen gene of a temperature sensitive mutant tsA58 of SV40 has been introduced.The immortalized cells are very useful because proliferation of the resulting cells and expression of the

differentiation character can be managed by changing the temperature (Noble M. et al. (1995) Transgenic Research 4, 215-225; Obinata M. (1997) Genes to Cells 2, 235-244). Rats having a body weight about ten times that of mice are advantageous for preparing cells used for the establishment of a cell line from various organs, particularly for preparing a cell line originating from small organs such as retinal capillary endothelial cells, because primary culture cells can be easily obtained by separating organs. Therefore, transgenic rats into which a large T-antigen gene of a temperature sensitive mutant tsA58 of SV40 has been introduced, which are useful for establishing immortalized cells due to easy availability from various organs and the capability of controlling the proliferation of the resulting cells and expression of the differentiation character by changing temperatures, had already been produced.

【0004】

On the other hand, in studies on blood retinal barrier a method of using a primary culture cell of retinal capillary endothelium in place of animal tests is being developed in view of animal right. In this instance, because it is difficult to obtain a sufficient amount of primary culture cells which can be provided for test from small animals, the eyeballs of large animals such as cattle must be used. However, the number of cells obtained by isolating retinal capillary endothelial cells from twenty eyeballs of cattle and subculturing the cells for two generations is at most $9x10^6$ or so (Wong H. C. et al. (1987) Invest. Ophthalmol, Visual, Sci.,

28, 1767-1775). Thus, a great number of eyeballs of cattle is required for screening drugs. Therefore, an effective retinal capillary endothelial cell stock which can be used in place of the cells from the eyeballs of cattle has been desired.

【0005】

【Problems to be Solved by the Invention】

In view of this situation, the present inventors have conducted extensive studies and, as a result, have established immortalized cells from transgenic rats into which immortalizing genes have been introduced by separating retinal capillary vessels from the retinal tissue of the rats and isolating retinal capillary endothelial cells from the resulting capillary vessels. An object of the present invention is therefore to obtain established cells derived from retinal capillary endothelial cells and capable of expressing a temperature sensitive SV40 large T-antigen, GLUT-1 transport carrier, and p-glycoprotein.

Another object of the present invention is to provide a method of establishing immortalized cells using a large T-antigen gene of SV40 temperature sensitive mutant tsA58.

【0006】

【Means to Solve the Problems】

The present invention relates to the established cells derived from retinal capillary endothelial cells. In particular, the present invention relates to established cells which express a temperature sensitive SV40 large T-antigen, GLUT-1 transport carrier, and p-glycoprotein. Cell deposited in National Institute of Bioscience and Human-Technology,

Agency of Industrial Science and Technology, the Ministry of International Trade and Industries, under the deposition number FERM BP-6507 can be given as such established cells.

Furthermore, the present invention relates to a method of establishing immortalized cells comprising homogenizing the retinal tissue of such a transgenic animal into which immortalizing genes have been introduced, separating capillary vessels, treating the resulting retinal capillary vessels with protease, and subculturing the resulting cells.

Furthermore, the present invention relates to the established cell obtained using such a method of establishment.

Such established cells of the present invention form a monolayer of the retinal capillary endothelial cells which have inside-and-outside polarity when culturing in culture dish. Therefore, the established cells are useful for predicting permeation of drugs into the retina by the assessment of drug uptake into the retinal capillary endothelial cells, studying supply and metabolism of various factors and nutritions in the retinal parenchyma, studying the transport mechanism of permeation of selective materials which are present in retinal capillary endothelial cells, studying toxicity of drugs on retinal capillary endothelial cells, and so on. In addition, a blood retinal barrier can be reconstructed in a test tube (in vitro) by coculturing with Mueller cells which are a kind of glia cells. The cell lines of the present invention are therefore useful in screening drugs regarding safety and efficacy thereof, and developing a method for diagnosing and treating diseases relating to

intraocular homeostatic maintenance and functional disorders of retinal tissues through cellular level studies.

【0007】

【Embodiments of the Invention】

The transgenic rat using in the present invention into which a large T-antigen gene of SV40 temperature sensitive mutant tsA58 has been introduced can be obtained as follows. Specifically, a whole genome DNA of tsA58ori(-)-2 which is produced from a large T-antigen gene of a temperature sensitive mutant tsA58 of SV40, for example, with deletion of the SV40 ori (replication origin), is linearized using a restriction endonuclease BamHI, and introduced into pBR322 to obtain a plasmid pSVtsA58ori(-)-2 (Ohno T. et al., Cytotechnology 7, 165-172 (1991)). The plasmid is amplified in Escherichia coli in a large amount according to a conventional method. The plasmid thus obtained is cut with a restriction endonuclease BamHI to eliminate a vector region. Because the DNA (5,240 bp) having a large T-antigen gene of tsA58 thus obtained has a promoter of the large T-antigen gene therein, a rat into which the DNA is introduced expresses this gene (the large T-antigen gene of tsA58) in all somatic cells.

【0008】

Next, the resulting DNA is introduced into totipotent cells of rats in accordance with a conventional method to prepare transgenic rats having a temperature sensitive large T-antigen gene in all cells. As a totipotent cell, ES cells having totipotency can be given in addition to fertilized ova and early embryos. A microinjection method, electropolation method, liposome metho

d, calcium phosphate method, and the like can be used for introducing DNA into such ova and cultured cells.

Furthermore, the present gene can be introduced into ova by transplanting a nucleus of cultured cells, into which a desired gene of the present invention has been introduced, in enucleation unfertilized ova and initializing the ova (nuclear transplantation). However, as far as the efficiency of obtaining a transgenic rat is concerned, a transgenic rat having a large T-antigen gene of tsA58 incorporated into chromosomes of cells of each tissue at the time of development of individuals can be efficiently obtained by producing ova through microinjection of the gene of the present invention into male pronucleus of the pronucleus fertilized ova, transplanting the ova into the oviduct of an foster mother to obtain offspring, and selecting the offspring having the injected gene, thereby stably obtaining individuals into which the gene of the present invention has been incorporated.

【0009】

Immortalized cells can be prepared by extracting cells (primary cells) from organs of gene-introduced rats thus obtained, and repeating subculture of the cells according to a conventional method. The resulting cells have the capability of permanently proliferating at 33-37°C and terminating the proliferation at 39°C, thus the cells have advantages that can control the expression of the differentiation character originally possessed by the cells. The retina is prepared from the eyeballs of this rat and cut into small pieces. The tissues are homogenized by using a taper-type homogenizer made of Teflon and the resulting slurry was centrifuged to obtain

pellets. The resulting pellets are suspended in an enzyme (protease) solution and treated with the enzyme while shaking, thereby separating capillary vessels from unnecessary tissues. Pellets are obtained by centrifugation. The pellets thus obtained are suspended in a Hanks' balanced salt solution (HBSS) containing 25% bovine serum albumin to remove unnecessary tissues. Capillary vessel pellets are recovered by centrifugation. After the capillary vessels are cut into fine pieces by enzyme treatment of the pellets by suspending again in the enzyme solution, the cells are inoculated in a culture dish. After subculturing two generations, colonies are formed. Colonies exhibiting a comparatively fast growth rate are isolated from surrounding cells using a penicillin cup. This procedure are repeated twice to isolate the cells of the present invention. Expression of a large T-antigen of tsA58, GLUT-1 transporter and p-glycoprotein are confirmed by the Western Blotting method, whereby the isolated cells are identified to be the immortalized retinal capillary endothelial cells. The cells thus obtained exhibit excellent growth after 50 generation subculture at 33°C and possess functions of retinal capillary endothelial cells.

【0010】

【Example】

The present invention will now be described in more detail by way of examples, which are given for the purpose of explanation and should not be construed as limiting the present invention.

【0011】

【Example 1】

Preparation of transgenic rat

A transgenic rat carrying DNA of an SV40 temperature sensitive mutant tsA58 was prepared according to the following method.

①Preparation of a gene to be introduced

DNA of SV40 temperature sensitive mutant tsA58 was used for microinjection. The genome DNA of tsA58 was linearized using a restriction endonuclease BamHI and introduced into the BamH site of pBR322 to convert the Sfi I sequence to the SacII sequence, thereby obtaining a DNA clone pSVtsA58 ori(-)-2 with deletion of the SV40 ori site (replication origin)(See Ohno T. et al., Cytotechnology 7, 165-172 (1991), Figure 1). The DNA was prepared from the pSVtsA58 ori(-)-2 according to a conventional method. Specifically, the pSVtsA58 ori(-)-2 of plasmid DNA obtained by amplification in Escherichia coli. was digested using a restriction endonucleases BamHI (made by Takara Shuzo Co., Ltd.) and DNA (Linear DNA fragment) of tsA58 with a length of 5240 bp separated the vector region by agarose gel electrophoresis (1% gel; Boeringer company)were cut out from the gel. The gel was dissolved by agarase treatment (0.6 unit/100 mg gel: Agarase; made by Boeringer Co.). DNA was recovered by phenol-chloroform treatment and ethanol precipitation treatment. The recovered and purified DNA was dissolved in a TE buffer (10 mM Tris-HCl containing 1 mM EDTA, pH 7.6) to obtain a purified DNA solution with a concentration of 170 μg/mL. The DNA solution was diluted with a buffer (10 mM Tris-HCl containing 0.1 mM EDTA, pH 7.6) to a

concentration of 5 μg/mL to prepare a DNA solution for microinjection. The resulting DNA solution was stored at -20°C until use for microinjection.

【0012】

②Preparation of transgenic rat

Preparation of transgenic rat Microinjection of the DNA solution prepared in ① above to the rat fertilized ova at pronucleus stage was carried out according to the following procedures. Sexually mature Wistar rats, aged eight weeks, were kept in a condition of a 12 hour light-and-shade cycle (light hours: 4:00-16:00) at 23±2°C and RH 55±5%. The estrous cycle of female rats was observed by vaginal smear to select the hormonal treating day. A pregnant-mare serum gonadotropic hormone (pregnant mare serum gonadotropin; PMSG, manufactured by Nippon Zenyaka Co.) was intraperitoneally administered at a dose of 150 IU/kg to female rats. After 48 hours, 75 IU/kg of human chorionic gonadotropic hormone (human chorionic gonadotropin; hCG, manufactured by Sankyo Zoki Co.) was administered thereby effecting superovulation treatment. The female and male rats were mated by being together in a cage. The fertilized ova at pronucleous stage were collected by oviduct perfusion at 32 hours after the hCG administration. A mKRB solution (Toyoda Y. and Chang M.C., J. Reprod. Fertil., 36, 9-22 (1974)) was used for the oviduct perfusion and incubation of ova. The collected (fertilized) ova were treated by an enzyme in an mKRB solution containing 0.1% hyaluronidase (Hyaluronidase Type I-S, made by Sigma Co.) at 37°C for 5 minutes to remove cumulus cells. After washing three times

with the mKRB solution to remove the enzyme, the fertilized ova were stored in a CO_2 incubator (5% CO_2-95% air, 37°C, saturated humidity) until DNA microinjection. A DNA solution was microinjected into the male pronucleus of the rat (fertilized) ova thus prepared. 228 ova after microinjection were transplanted in nine recipients (foster mothers) and 80 pups were obtained.

The integration of the microinjected DNA was analyzed with DNA p repared from tails of the rats immediately after weaning by the PCR method (primers used: tsA58-1A, 5'-TCCTAATGTGCAGTCAGGTG-3' (corresponds to 1365-1384 sites), tsA58-1B, 5'-ATGACGAGCTTTGGCA CTTG-3' (corresponds to 1571-1590 sites)). As a result, 20 rats (6 male, 8 female, and 6 unknown sexuality) were identified to have the gene introduced. Among these rats, 11 transgenic rat l ines (male lines: #07-2, #07-5, #09-6, #12-3, #19-5, female line s: #09-7, #11-6, #12-5, #12-7, #18-5, #19-8) which survived as l ong as 12 weeks after elapse of the sexual maturation period wer e obtained. These G_0 generation transgenic rats were mated with Wistar rats and established 2 lines of male founders (#07-2, #0 7-5) and 3 lines of female founders (#09-7, #11-6, #19-8), by co nfirming that the genes was transferred to next generation.

【0013】

【Example 2】

<u>Isolation of retinal capillary endothelial cells from transgenic rats</u>

The method of Greenwood (Greenwood J. (1992) J. Neuroimmun., 39, 123-132) was modified and applied to the isolation of retinal capillary endothelial cells from the

retina. Eyeballs were collected from one transgenic rat carring a large T-antigen gene of SV40 temperature sensitive mutant tsA58 described in Example 1. The eyeballs were thoroughly washed with an ice-cooled buffer solution (HBSS containing 10 mM Hepes, 100 U/mL benzylpenicillin potassium, 100 μ/mL streptomycin sulfate, 0.5% bovine serum albumin) in a clean bench. The retinal tissue was removed and cut into pieces with a volume of 1-2 mm^3. The tissue pieces were placed in a 1 mL taper-type Teflon homogenizer (WHEATON Co.). 1 mL of ice-cooled buffer solution was added and the tissue was homogenized by four up-and-down strokes to obtain a slurry. The resulting slurry was centrifuged (600 g, 5 minutes, 4°C) to obtain pellets. The pellets were suspended in a 1 mL of enzyme solution (HBSS containing 0.01% collagenase/dispase (Boehringer Mannheim) 100 U/mL benzylpenicillin potassium, 100 μ/mL streptomycin sulfate, 20 U/mL deoxyribonuclease I, 0.147 μ/mL tosyl-lysine-chloromethylketone) and digested by enzyme in a water bath with shaking at 37°C for 30 minutes, thereby separating capillary vessels from unnecessary tissues. The enzyme treated slurry was centrifuged (600 g, 5 minutes, 4°C) to obtain pellets.

【0014】

The pellets thus obtained were suspended in HBSS containing 25% bovine serum albumin to remove unnecessary tissues. The pellets of capillary vessel fraction were obtained by centrifugation (1,000 g, 15 minutes, 4°C). The pellets were suspended again in a 1 mL enzyme solution and treated at 37°C for 30 minutes to digest the capillary vessels

into fine pieces. The enzyme treated slurry was centrifuged (600 g, 5 minutes, 4°C) to obtain pellets. Next, the pellets obtained were dispersed in a 2 mL culture solution (DMEM containing 15 μg/mL endothelial cell growth factor, 100 U/mL benzylpenicillin potassium, 100 μ/mL streptomycin sulfate, 2.50 μ/mL amphotericin B) and inoculated in a 35 mmØ culture dish coated with collagen type I (a product of Becton Dickinson Co.). The cells were incubated (primary culture) at 33°C in a CO_2 incubator (5% CO_2-95% air, saturated humidity). Subculture was carried out at an interval of about one week using a trypsin solution (0.05% Trypsin, 0.53 mM EDTA; manufactured by Gibco BRL) while replacing the medium twice a week. After subculturing twice, 10^2-10^3 cells were inoculated in a 100 mmØ culture dish coated with collagen type I (a product of Becton Dickinson Co.). The cells were incubated at 33°C in a CO_2 incubator to form colonies. After preparation of colonies for 7-10 days while replacing the medium twice a week, the colonies exhibiting a comparatively fast growth rate were isolated from the surrounding cells using a penicillin cup. The cells obtained were again inoculated in a 100 mmØ culture dish and incubated at 33°C in a CO_2 incubator to form colonies. Colonies exhibiting a comparatively fast growth rate were isolated using a penicillin cup to obtain five lines of cells (TR-iBRB2, TR-iBRB4, TR-iBRB6, TR-iBRB8, TR-iBRB9).

TR-iBRB2 was deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technolo gy, the Ministry of International Trade and Industries. The dep osition number is FERM BP-6507.

【0015】

【Example 3】

Confirmation of large T-antigen proteins

Expression of large T-antigen proteins in the five cell lines obtained in Example 2 was examined by the Western Blotting method (Experimental Medicine Separate Volume, Biotechnology Manual UP Series, "Cancer research protocol by the molecular biological approach", pages 108-115, YODOSHA Publishing Co., 1995). The five cell lines (the 20th generation) were cultured in 90 mmØ culture dishes until saturation. The collected cells were solubilized using 3% SDS-PBS (pH 7.4) and unsolubilized fractions were removed by centrifugation (10,000 rpm, 10 minutes), and then the total amount of proteins was determined by the Bradford method (using the protein assay kit II of the BIO-RAD Co.). The proteins were separated by the SDS polyacrylamide gel electrophoresis in the amount of 20 μg each and transferred onto nitrocellulose membranes. The nitrocellulose membranes blocked by a 3% skimmed milk solution were reacted with an anti-SV40 large T-antigen mouse antibody (DP02-C, CALBIOCHEM Co.), as a primary antibody, and a HRP labeled anti-mouse IgG antibody (Amersham Co.), as a secondary antibody, to detect the reactions specific to large T-antigen proteins by using the ECL Western Blotting detection system (RPN2106M1) made by Amersham Co.. The results are shown in Table 1. In the Table, "+" indicates that the reaction specific to a large T-antigen protein was detected. As a result, the expression of large T-antigen proteins was confirmed in all five cell lines.

【0016】

【Table 1】

Cells	TR-iBRB2	TR-iBRB4	TR-iBRB6	TR-iBRB8	TR-iBRB9
T-Antigen	+	+	+	+	+

【0017】

【Example 4】

Identification of cell lines

The cell lines obtained in Example 2 were identified to be retinal capillary endothelial cells by confirming the expression of a GLUT1 transporter and p-glycoprotein by the Western Blotting method. Using nitrocellulose membranes prepared in the same manner as in Example 3, the cells obtained were reacted with an anti-GLUT-1 mouse antibody (Temecular, CA, Chemicon Co.) or an anti-p-glycoprotein rabbit antibody (anti-mdr antibody, Oncogene Research Products Co.), as primary antibodies, and a HRP labeled anti-mouse IgG antibody (Amersham Co.) or a HRP labeled anti-rabbit IgE antibody (Cappel Co.), as secondary antibodies, to detect the reactions specific to GLUT-1 protein or p-glycoprotein using the ECL Western Blotting detection system (RPN2106M1) made by Amersham Co.. The results are shown in Table 2. The results are shown in Table 2. In the Table, "+" means that the GLUT-1 protein or p-glycoprotein were detected. As a result the GLUT-1 protein and p-glycoprotein were detected in all five cell lines. Therefore, the five cell lines obtained were detected to be retinal capillary endothelial cells.

【0018】

【Table 2】

Cells	TR-iBRB2	TR-iBRB4	TR-iBRB6	TR-iBRB8	TR-iBRB9
T-Antigen	+	+	+	+	+
P-Glycoprotein	+	+	+	+	+

【0019】

【Example 5】

Confirmation of glucose transport capability

Using the cells TR-iBRB2 obtained in Example 2, The 3-OMG (3-o-methyl-D-glucose) uptake capability was determined to confirm that the cells have a functional GLUT-1 transporter from exhibiting concentration-dependent glucose transport capability. Specifically, TR-iBRB cells were inoculated in a 24-well cell culture plate at a concentration of $3x10^5$ cells /well/mL and incubated for 24 hours at 33°C in a CO_2 incubator to make the cells confluent. The 3-OMG uptake was determined according to the following procedure. After removing medium by aspiration, 0.2 mL of an uptake buffer containing 232 kBq/mL of $[^3H]$3-OMG heated to 37°C was added. The uptake buffer used in this Example did not contain glucose and was prepared from a solution which contains 122 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM K_2HPO_4, 10 mM Hepes, and 25 mM $NaHCO_3$ by bubbling 5% CO_2-95% O_2 into the solution for 20 minutes and adjusting the pH of the resulting solution to 7.4 with NaOH (this is hereinafter designated as uptake buffer ①). After 10 seconds, the uptake buffer ① was removed and the residue was washed with the uptake buffer ① having 4°C of temperature. The

same procedure was then repeated except for changing the period of time removing the uptake buffer ① to 20 seconds, 30 seconds, or one minutes. The cells were solubilized overnight in 1 mL of PBS containing 1% Triton X-100 and the radioactivity was measured using a liquid scintillation counter to confirm the linearity of the 3-OMG uptake capability. As a result, an uptake time of 20 seconds was set.

【0020】

Next, the substrate concentration dependency of the 3-OMG uptake capability was examined. After washing the cells with the uptake buffer ① heated to 37°C, 0.2 mL of the uptake buffer ① containing 462 kBq/well of [^{3}H]3-OMG heated to 37°C was added.

Solutions containing 3-OMG at different concentrations were prepared by using the uptake buffer ① containing non-labeled 3-OMG at a concentrations of 0, 0.5, 1, 5, 10, 20, 30, and 50 mM. After 20 seconds, the uptake buffer ① was removed and the residue was washed with the uptake buffer ① containing 10 mM non-labeled 3-OMG at 4°C. Next, the cells were solubilized overnight in 1 mL of PBS containing 1% Triton X-100 and the radioactivity was measured using a liquid scintillation counter. The results are shown in Figure 1. Using the plot formula for the uptake rate vs. the 3-OMG concentration (V = Vmax X [S]/(Km + [S]), wherein Vmax indicates a maximum velocity constant, Km indicates the Michaelis constant, and [s] is a substrate concentration), the Km and the Vmax for 3-OMG uptake were analyzed using the non-linear minimum square program (Yamaoka K.et al. (1981) J. Pharmacobio-Dyn., 4, 879-

885). As a result, it was confirmed that the uptake of [^{3}H]3-OMG which is the substrate of the GLUT-1 was concentration-dependent, the Michaelis constant (Km) was 5.6 mM, and the maximum velocity constant (Vmax) was 45 nmol/min/mg protein. Accordingly, the cell lines of the present invention were confirmed to exhibit a concentration-dependent glucose transport capability.

【0021】

【Example 6】

Transport capability of p-glycoprotein

Possession of a functional p-glycoprotein transport capability by cells TR-iBRB2 obtained in Example 2 was examined by measuring the uptake of cyclosporin A (CyA) which is the substrate of the p-glycoprotein and comparing the results with the uptake capability under the presence of verapamil which is a p-glycoprotein inhibitor. Specifically, cell lines TR-iBRB were inoculated in a 24-well cell culture plate at a concentration of $1x10^5$ /well/mL culture medium and incubated at 33°C in a CO_2 incubator to make the cells confluent. The CyA uptake was determined according to the following procedure. After removing the medium by aspiration, the cells were washed with an uptake buffer previously heated to 37°C containing glucose (which was prepared from a solution which contains 122 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM K_2HPO_4, 10 mM Hepes, 25 mM $NaHCO_3$, and 10 mM D-glucose by bubbling 5% CO_2-95% O_2 into the solution for 20 minutes and adjusting the pH of the resulting solution to 7.4 with NaOH; this is hereinafter designated as uptake buffer ②). After the addition of 0.2 mL of uptake buffer ② heated to 37°C containing 0.

25% DMSO, the cells were preincubated for 30 minutes. Then, the uptake buffer ② was removed and 0.2 mL of uptake buffer ② containing 37 kBq/mL of [^{3}H]CyA, 0.075 μM of non-labeled CyA, and 0.25% DMSO, heated to 37°C, was added. For uptake in the presence of verapamil, 0.2 mL of uptake buffer ② containing 100 μM of verapamil and 0.25% DMSO heated to 37°C was added, and the cells were preincubated for 30 minutes, followed by the removal of the uptake buffer ② and the addition of 0.2 mL of uptake buffer, heated to 37°C, containing 37 kBq/mL [^{3}H]CyA, 0.075 μM non-labeled CyA, 100 μM verapamil, and 0.25% DMSO. Both uptake reactions were carried out for 30 minutes. After removing the reaction solution, the residue was washed with uptake buffer ② of 4°C three times, and then cells were solubilized overnight with the addition of 1 mL of 1N NaOH. Then, the radioactivity was determined using a liquid scintillation counter. As a result, a significant increase in the uptake amount of about 1.8 times was confirmed. Specifically, the cell/medium uptake ratio of [^{3}H]CyA which is the substrate of the p-glycoprotein was 270 μL/mg protein, whereas the cell/medium uptake ratio of [^{3}H]CyA in the presence of 100 μM verapamil which is an inhibitor of the p-glycoprotein was 490 μL/mg protein. The same results were obtained with other cell lines.

【0022】

【Example 7】

Confirmation of function of scavenger receptor

Possession of a functional scavenger receptor in cells of TR-iBRB2 obtained in Example 2 was examined by measuring

uptake of an acetylated LDL (Dil-Ac-LDL, Biomedical Technologies, Stoughton, MA) labeled with a fluorescence labeling material, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate. Specifically, TR-iBRB2 cells were inoculated on a cover glass at a concentration of $1x10^5$ /well/mL medium and incubated at 33°C in a CO_2 incubator for 48 hours to make the cells confluent. The Dil-Ac-LDL uptake was determined according to the following procedure. After removing the medium by aspiration, the cells were washed with the uptake buffer ② previously heated to 37°C. Next, 0.2 mL of the uptake buffer ② containing 10 μg/200 μL Dil-Ac-LDL which was heated to 37°C was added, followed by incubation in a CO_2 incubator for 30 minutes. After 4 hours, the uptake buffer ② was removed and the residue was washed with the uptake buffer ② of 4°C. After the addition of 3% formaldehyde/PBS and immobilization by keeping at room temperature for 20 minutes, fluorescence uptaken into cells were measured by using a confocal laser scanning microscopy. As a result, uptake of an acetylated LDL (Dil-Ac-LDL) labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, which is a scavenger receptor ligand, into the cells was confirmed. The same results were obtained with other cells.

【0023】

【Effects of the Invention】

The present invention provides established cells originating from retinal capillary endothelial cells, which express a temperature sensitive SV40 large T-antigen, GLUT-1 transporter, and p-glycoprotein. Furthermore, a method of

establishing immortalized cells can be provided, which comprises homogenizing the retinal tissue of a transgenic animal carring a large T-antigen gene of SV40 temperature sensitive mutant tsA58, separating capillary vessels, treating the resulting retinal capillary vessels with protease, and subculturing the resulting cells.

Such established cells obtained by the present invention form a monolayer of the retinal capillary endothelial cells which have inside-and-outside polarity when culturing in culture dish. Therefore, the established cells are useful for predicting permeation of drugs into the retina by the assessment of drug uptake into the retinal capillary endothelial cells, studying supply and metabolism of various factors and nutritions in the retinal parenchyma, studying the transport mechanism of permeation of selective materials which are present in retinal capillary endothelial cells, studying toxicicology of drugs on retinal capillary endothelial cells, and so on. In addition, a blood retinal barrier can be reconstructed in a test tube (in vitro) by coculture with Mueller cells which are a kind of glia cells. The cell strains of the present invention are therefore useful in screening drugs regarding safety and efficacy thereof, and developing a method for diagnosing and treating diseases relating to the maintenance of intraocular homeostasis and functional disorders of retinal tissues on the cellular level studies.

【Brief Description of the Drawings】

【figure 1】

Figure 1 shows a substrate concentration dependency of a 3-OMG uptake speed in Example 5.

JP No 10-296138

【Document Name】 Abstract

【Abstract】

【Problems】 To provide established cells.

【Means to be solved】

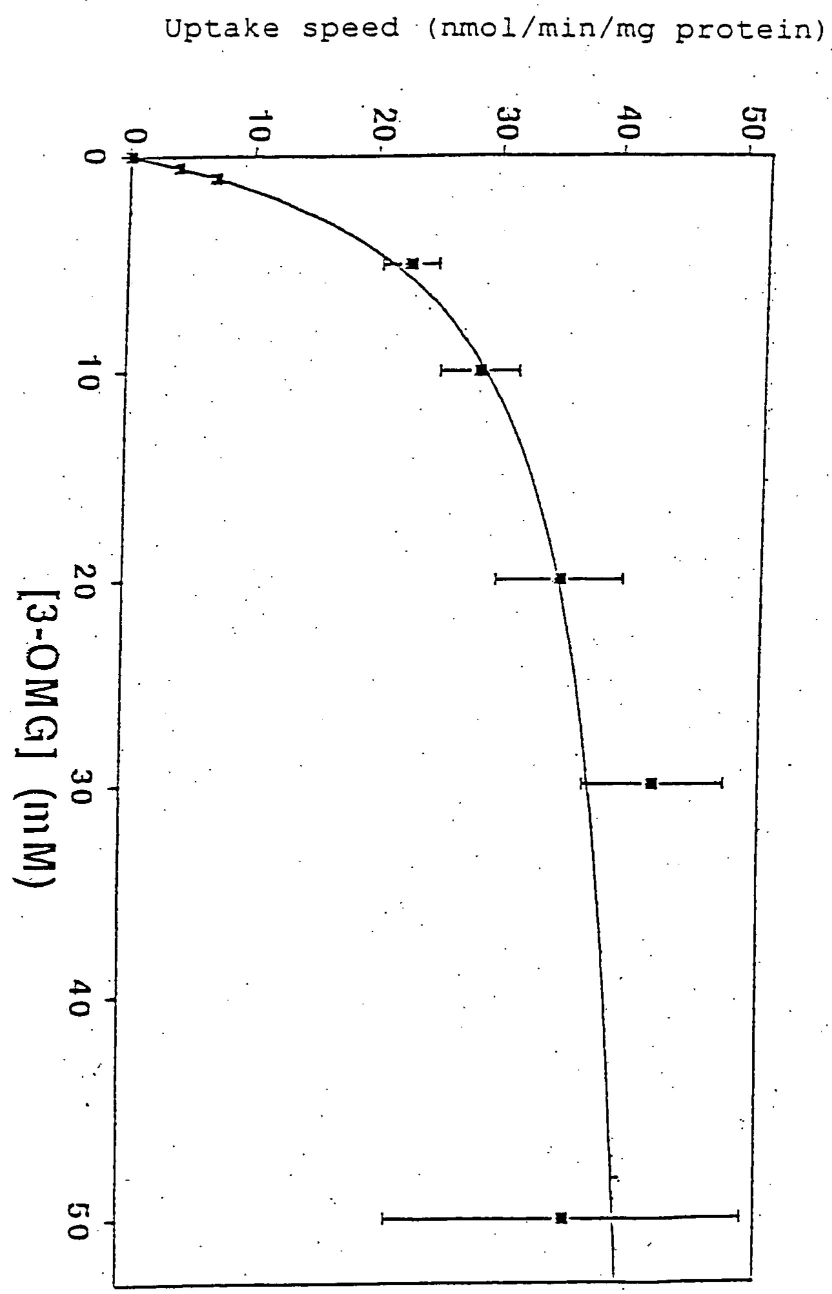
Eestablished cells originating from retinal capillary endothelial cells, which express a temperature sensitive SV40 large T-antigen, GLUT-1 transporter, and p-glycoprotein.

A method of establishing immortalized cells can be provided, which comprises homogenizing the retinal tissue of a transgenic animal carring a large T-antigen gene of SV40 temperature sensitive mutant tsA58, separating capillary vessels, treating the resulting retinal capillary vessels with protease, and subculturing the resulting cells.

【Effect】 These cells are useful in screening drugs regarding safety and efficacy thereof, and developing method for diagnosing and treating diseases relating to nutrition metabolism in retinal tissues and brain on cellular level studies.

【Drawing】 Non

【 Figure 1 】



整理番号 SNMFP98378

国際様式　INTERNATIONAL FORM

1／1

〔特許手続上の微生物の寄託の国際的承認に関するブダペスト条約〕

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

下記国際寄託当局によって規則7．1に従い発行される。

原寄託についての受託証

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

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1．微生物の表示	
（寄託者が付した識別のための表示） TR－iBRB2	（受託番号） FERM BP－ 6507

2．科学的性質及び分類学上の位置

1欄の微生物には、次の事項を記載した文書が添付されていた。

- ☐ 科学的性質
- ■ 分類学上の位置

3．受領及び受託

本国際寄託当局は、　平成 10 年　 9 月　18 日（原寄託日）に受領した1欄の微生物を受託する。

4　移管請求の受領

本国際寄託当局は、　　　年　　月　　日（原寄託日）に1欄の微生物を受領した。
そして、　　　年　　月　　日 に原寄託よりブダペスト条約に基づく寄託への移管請求を受領した。

5．国際寄託当局

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